Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 1128–1132, February 1992
Developmental Biology

Onset of cell-specific gene expression in the developing mouse pancreas

(pancreas development/PCR/cell lineages/organogenesis)

GEORGE K. GITTES* AND WILLIAM J. RUTTER†‡

*Department of Surgery and †The Hormone Research Institute, University of California, San Francisco, CA 94143

Contributed by William J. Rutter, November 5, 1991

ABSTRACT **A central question in developmental biology has been the initiation of cell-specific gene expression and its temporal relationship to morphogenesis. We have coupled embryo microdissection with the exquisite sensitivity of the polymerase chain reaction to define the onset of cell-specific gene expression during pancreatic organogenesis. Using the precise assignment of gestational age by the number of somites in each embryo, we determined the onset of transcription of major genes of the endocrine and exocrine pancreas during mouse development to within 2–3 hr. Somatostatin mRNA was detected at the 10-somite stage throughout the foregut, consistent with the presence of somatostatin-producing cells throughout the adult gut. Mature mRNA for insulin and glucagon first appears surprisingly early, at the 20-somite stage in the wall of the embryonic foregut and is restricted to only the area of the duodenum from which the pancreas will arise 10–12 hr later. In contrast, exocrine gene transcription begins 24 hr after formation of the pancreatic diverticulum. Thus cell-specific gene expression in the endocrine pancreas begins in a "premorphogenetic phase." This early expression of insulin and glucagon could reflect the initiation of an endocrine cell lineage.**

Organogenesis in the embryo consists of two phases: (*i*) morphogenesis, the development of multicellular structures characteristic of a specific organ, and (*ii*) cytodifferentiation, the expression of the organ-specific, differentiated cellular phenotype. It has been presumed that morphogenesis precedes cell-specific gene expression (1, 2). Morphogenesis of the pancreas begins by evagination of the duodenum at 26 somites (day 9.5 of gestation in the mouse, Fig. 1; and day 10.5 in the rat) (3, 4). Cytodifferentiation and cell-specific gene expression have been studied extensively by Northern hybridization (using specific cDNA probes) (5), by immunohistochemistry (6), and by enzymatic activity (7). These studies in both rat and mouse first detected glucagon transcription and translation 0.5–1 day after the evagination of the pancreas (5, 8) and insulin about 1 day later (5, 9). Exocrine gene expression was detected later than both glucagon and insulin: carboxypeptidase on day 12 and amylase on day 13 (in rat) (5). Other islet hormones were detected even later: somatostatin on day 16 and pancreatic polypeptide on day 20 (in mouse) (6).

Previous studies of developmental expression have employed techniques that are somewhat limited in sensitivity and specificity. For mRNA detection, for example, neither Northern hybridization nor *in situ* hybridization is sensitive enough to detect the few molecules of mRNA that may represent the earliest evidence of differentiation during organogenesis. Reverse transcriptase-PCR (RT-PCR), however, which does allow detection of such a small number of molecules (10), does not provide any spatial information about mRNA expression. In our experiments we separately dissected specific regions of the developing foregut and pancreas for assay by RT-PCR (Fig. 1) so that the early, low-level gene expression during the formation of the pancreas could be delineated. A second problem with many previous studies of developmental expression is the definition of gestational age of the embryo. Commonly noon of the date of vaginal plug is assumed to be gestational age 0.5 days. This assumption entails an error of ±8 hr. Instead we counted the number of somites in each embryo and thereby determined the onset of pancreas-specific gene expression to within 2–3 hr (probably less than the time of one cell division).

Our results indicate that there is transcription of endocrine pancreas-specific genes in the foregut prior to pancreas formation. In addition, this expression is localized to the area of foregut from which the pancreas will arise some time later. This "premorphogenetic phase" of organogenesis may represent the initiation of endocrine cell lineages.

MATERIALS AND METHODS

Embryonic Tissue Procurement. Male and female B6D2F$_1$ mice from Simonson Laboratories (Fremont, CA) were mated overnight. The presence of a vaginal plug was indicative of pregnancy. For rough approximation of gestational age, noon of the day of vaginal plug was considered to be gestational age 0.5 day. At serial gestational ages, pregnant females were sacrificed, and embryonic pancreases or foreguts were dissected in Hanks' balanced salt solution by using a Zeiss ZEMI SV8 ×64 dissecting stereomicroscope. The precise age of the embryo was determined by counting somites. Tissue samples were placed in guanidinium thiocyanate/2-mercaptoethanol, frozen in liquid N_2, and stored at −80°C prior to RNA extraction.

cDNA Preparation and PCR. RNA was extracted from tissues by a method adapted from Rappolee *et al.* (10). Individual tissue specimens in guanidinium thiocyanate/2-mercaptoethanol, with 20 μg of added *Escherichia coli* rRNA as carrier, were sonicated at 4°C for 10 sec. Sonicated tissue was then layered over a 100-μl CsCl gradient and centrifuged at 360,000 × *g*. The pellet was resuspended in 100 μl of guanidinium thiocyanate/2-mercaptoethanol and centrifuged again over 100 μl of CsCl. Precipitation with ammonium acetate and ethanol yielded 40–80% of the original carrier. Reverse transcription with avian myeloblastosis virus reverse transcriptase and oligo(dT) was performed as described by Rappolee *et al.* (10). Ten percent of the cDNA was amplified by using a 40-cycle PCR with a 60°C annealing temperature. Twenty percent of the PCR product was then run on an ethidium bromide-stained agarose gel. Bands visible with UV illumination were considered positive.

To pinpoint the time of onset for a particular gene, an initial survey of time points over a wide range was performed,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RT-PCR, reverse transcriptase-PCR; HBV, hepatitis B virus.

‡To whom reprint requests should be addressed.

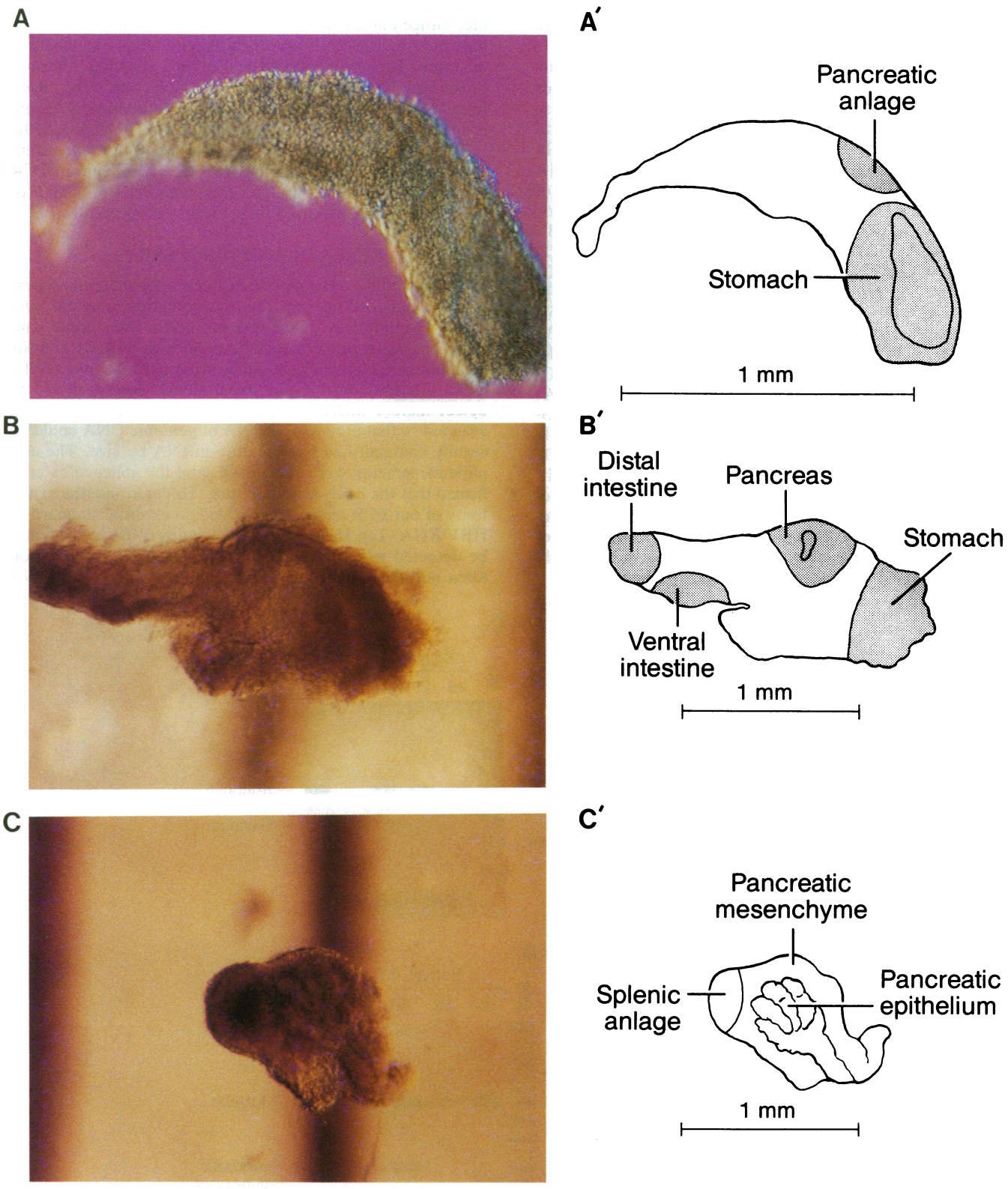

FIG. 1. Microdissection of the developing mouse foregut and pancreas. On the left is the tissue as viewed through the dissecting microscope. On the right is a corresponding schematic in which shaded areas were separately isolated and assayed by RT-PCR. (*A*) Twenty-somite stage foregut, shown here under Nomarski optics. The hepatic rudiment has been dissected away. (*A′*) The area of the incipient stomach with early lumen formation, as well as the duodenal anlage from which the pancreas will evaginate, are shown. (*B*) Twenty-six-somite stage. Note that the pancreas is first beginning to evaginate from the dorsal surface of the foregut. (*B′*) Different portions of the foregut were dissected and assayed separately, including pancreas, dorsal and ventral intestine in the vicinity of the pancreas, stomach, and distal intestine. (*C*) Forty-eight-somite pancreas. (*C′*) The pancreatic epithelium within the surrounding pancreatic mesenchyme is beginning to branch in the formation of early acini. The splenic anlage was removed prior to RT-PCR.

followed by narrower time points to cover the range in which initiation of expression occurred. Critical time points were repeated at least three times.

Primer Design. PCR was performed using pairs of primers situated so that genomic DNA and unspliced precursor mRNA, if present, would amplify as larger fragments because of intron(s). Twenty-mer oligonucleotides were designed to generate cDNA fragments between 250 and 650 base pairs. In the case of carboxypeptidase A and glucagon, the cDNA sequences in mouse are unknown so conserved sequences of related species were used. Specific primer pairs are as follows: β-tubulin, 5′-TGGCCAGATCTTCAGACCAG-3′ and 3′-GAGTGACACGGACTTGAATG-5′; insulin, 5′-CAGCCCT-TAGTGACCAGCTA-3′ and 3′-CTAGTCACGACGTG-GTCGTA-5′; glucagon, 5′-ATTCACAGGGCACAT-TCACC-3′ and 3′-GGTCTCTGAAGTAGTTGACC-5′; somatostatin, 5′-CCGTCAGTTTCTGCAGAAGT-3′ and 3′-GCTACGAGTTGAACTGGGAC-5′; pancreatic polypeptide, 5′-AGGATGGCCGTCGCATACTG-3′ and 3′-CTGAAG-GACCTCACGTCGAG-3′; carboxypeptidase A, 5′-GGCATCCATTCTAGGGAGTGG-3′ and 3′-GTCCCG-TAGTTAATRAGAAAG-5′; amylase, 5′-GTAGCAGGGT-TCAGACTTGA-3′ and 3′-ACGTCTGTGATGAACACCGT-3′. The insulin primer pair is degenerate for insulins I and II.

Fragment Identification. M13 forward and reverse sequencing signals were attached to primers for all six pancreatic primer pairs. PCR-amplified cDNA from the time of initial expression for each gene was then sequenced directly using an Applied Biosystems 370A DNA sequencer.

Sensitivity Determination. A hepatitis B virus (HBV) surface antigen plasmid was transcribed *in vitro* with SP6 RNA polymerase. The RNA was then heavily DNase treated and quantitated spectrophotometrically, thus allowing, after appropriate dilution, a small number of molecules to be added to the original tissue preparation in guanidinium thiocyanate prior to sonication. The minimum number of molecules of the HBV RNA required to be added to allow for consistent detection after RNA isolation, reverse transcription, PCR amplification of a 700-base-pair fragment, and ethidium bromide/agarose gel electrophoresis was ≈10 molecules.

RESULTS AND DISCUSSION

We measured the onset of expression of six pancreatic genes: insulin, somatostatin, pancreatic polypeptide, amylase, and carboxypeptidase A. RNA was purified from a single pancreas and assayed for a specific pancreatic mRNA and for β-tubulin mRNA (which should be present in all or most cells) as an internal control. The experiments were designed to detect mature mRNA; oligonucleotide primers were constructed so that precursor mRNA or genomic DNA could be readily distinguished from mature mRNA by size. The amplified fragments were sequenced, and the splice sites confirmed that the mRNA was mature. To determine the sensitivity of our PCR method, we added an external standard of HBV RNA. Ten HBV mRNA molecules could consistently be detected when added to the original tissue preparation (data not shown). Thus a single cell containing 10 mRNA

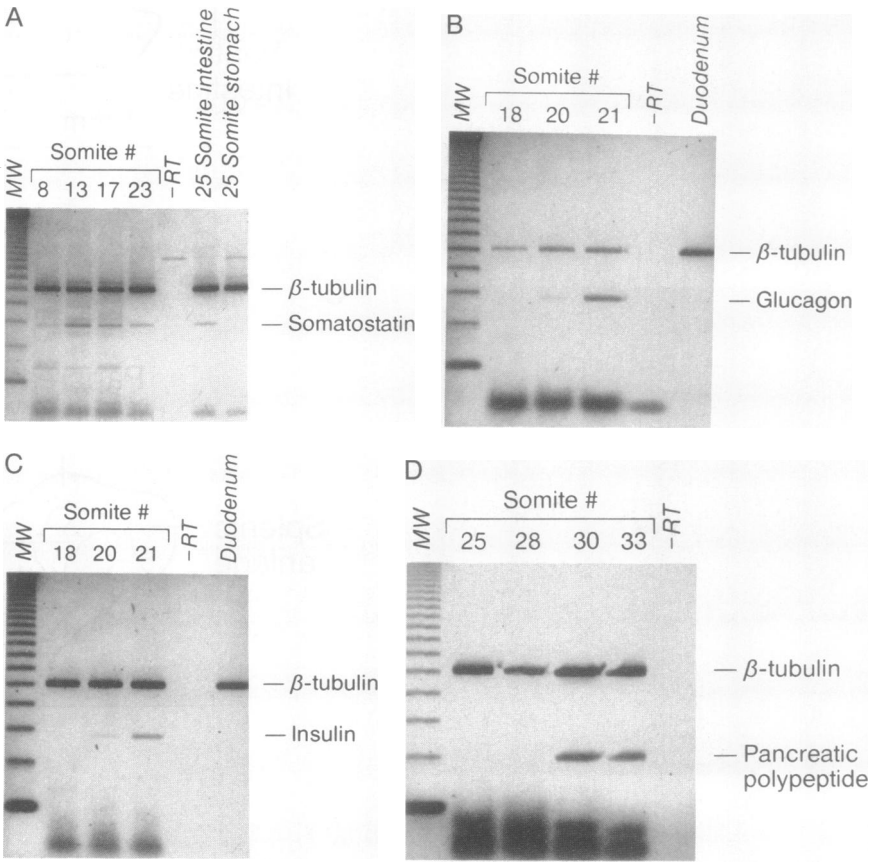

FIG. 2. PCR-amplified endocrine cDNA signals from individual embryonic foregut and pancreases at sequential gestational ages. Data are shown as reverse image ethidium bromide-stained agarose gels. β-Tubulin was used as an internal control. The −RT control is for the earliest positive somite stage. MW, molecular weight markers. (*A*) Somatostatin transcript is present in the earliest embryos dissected, at the 8-somite stage. This transcription is not, however, specific to the area that will form pancreas. (*B* and *C*) Glucagon and insulin transcript are both first detectable at the 20-somite stage, specifically in the area of intestine destined to form the pancreas. Adjacent duodenum (as well as stomach and distal intestine; data not shown) did not contain insulin or glucagon transcripts. Sequencing revealed that the majority of insulin was insulin I. (*D*) Pancreatic polypeptide transcript is first present at the 30-somite stage.

molecules, or 10 cells, each containing a single molecule, should be detected by this RT-PCR procedure.

By using these methods, the onset of gene expression was found to occur significantly earlier than previously reported. Somatostatin mRNA was detected in the foregut of the earliest embryos assayed (8 somites or ≈7.5 days); these transcripts were found not only in the area of duodenum from which the pancreas would eventually arise but also in the intestinal anlage not destined to form pancreas (Fig. 2*A*). This result is consistent with the presence of somatostatin-producing cells throughout the intestine of the mature animal (11).

In contrast to somatostatin, glucagon and insulin mRNA were first detected simultaneously at the 20-somite stage (roughly 8.5–9 days; Figs. 1*A* and 2 *B* and *C*), 10–12 hr before the condensation of the mesenchyme near the gastrointestinal junction (25 somites), an event that presages the evagination of the pancreatic diverticulum (Fig. 1*B*). Both insulin and glucagon transcripts were specifically localized to the dorsal wall of the duodenal anlage, in the precise area that gives rise to the pancreas. Immediately adjacent tissues such as the dorsal and ventral duodenum, stomach, and adjacent distal intestine did not contain insulin or glucagon transcripts at the time of pancreatic evagination. Thus, in contrast to the prevalent view that morphogenesis precedes organ-specific gene expression (2), the genetic program for the pancreatic islet α and β cells involves a "premorphogenetic phase" during which specific genes are activated prior to detectable morphogenetic events.

Pancreatic polypeptide transcripts were first detected at the 30-somite stage, 8–10 hr after evagination of the pancreas (Fig. 2*D*). At this stage the ventral pancreas wraps around the duodenal anlage to fuse with the dorsal pancreas (see Fig. 4). Pancreatic polypeptide is thought to be expressed selectively in the ventral pancreas (12). The data indicate that the expression of individual islet endocrine genes, as well as the cells that specifically express each of these genes, develop at different times in gestation.

In all cases the endocrine-specific transcripts were detected significantly earlier than the first detection of the corresponding protein by RIA (8, 9) or immunohistochemistry (6). This delay is, to some degree, caused by the extreme difference in sensitivity of the assays: RT-PCR is estimated to be at least 10^8 times more sensitive than protein assays. This discrepancy could also reflect the time necessary for accumulation of protein from RNA transcripts (i.e., approximately one protein molecule per minute per transcript). It is also possible that the early endocrine cells contain only the constitutive secretory pathway, and thus the synthesized peptide hormones would be directly secreted rather than stored in secretory granules inside the cell (and thus might be undetectable). Previous studies have shown that during embryogenesis the capacity to regulate insulin secretion develops considerably later than the capacity to synthesize insulin (13).

Transcription of exocrine genes, carboxypeptidase A and amylase, was initiated later than all of the endocrine genes and well after formation of the pancreatic rudiment: carboxypeptidase A was first detected at 36 somites (day 10.5) and amylase was first detected in the postsomite period, at gestational day 12 (Fig. 3). Since these genes are probably expressed in the same acinar cells, all of the exocrine genes for one cell are not activated simultaneously. This finding is consistent with previous studies on the levels of specific exocrine mRNAs during development (5). Previous methods (5, 7) were not as specific, however, because of the specific oligonucleotide probes employed in our experiments. Here PCR amplification presumably should detect only expression of the pancreatic amylase gene, not the related hepatic or salivary amylase genes (14).

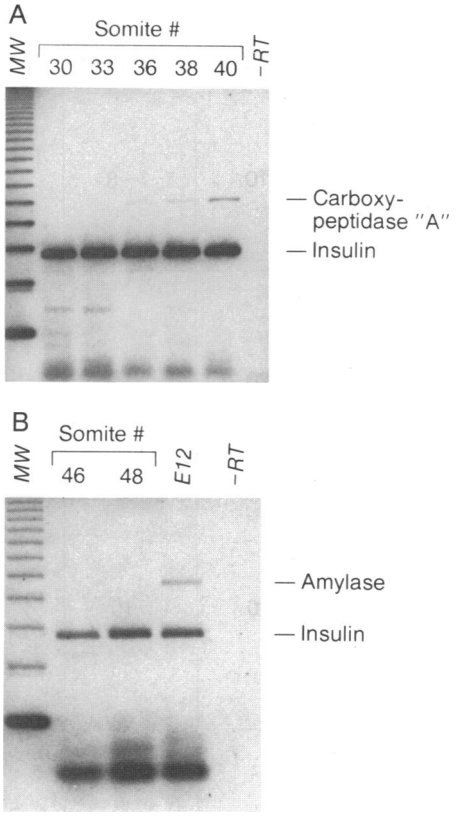

FIG. 3. PCR-amplified exocrine cDNA signals from individual embryonic pancreases at sequential gestational ages. Data are shown as reverse image ethidium bromide-stained agarose gels. Insulin is used as an internal control. The −RT control is for the earliest positive somite stage. MW, molecular weight markers. (*A*) The carboxypeptidase A transcript is first present at the 36-somite stage. (*B*) The amylase transcript is first present at embryonic day 12 (E12), during the early postsomite stage. Sequence data confirm the amylase as pancreatic amylase, not hepatic or salivary.

Fig. 4 summarizes the relationship of the onset of specific gene expression to concurrent morphogenetic events. The dorsal wall of the duodenal anlage contains cells that express insulin and/or glucagon, located specifically in the area from which the pancreas will arise. Hanahan and colleagues (15) have detected cells present in the early pancreas that can express both insulin and glucagon. It is possible that expression of these genes occurs in precursor cells that are involved in the evagination of the duodenum to form the pancreas. Assuming a doubling time of 8 hr, one of these progenitor cells expressing insulin and/or glucagon at the 20-somite stage could readily give rise to the 2000–10,000 islet cells present in the 12-day embryonic pancreas (3). Thus it is possible that an endocrine lineage develops from a single or a few progenitor cells.

Transcription of the exocrine-specific genes begins later, closer to the time when exocrine enzymes are first detectable by enzymatic activity (7). This relatively late expression might reflect a functional role of the endocrine cells in exocrine development. However, another physiological explanation seems attractive: the delay in expression of exocrine enzymes might prevent the autolysis that could result from the secretion and activation of digestive enzymes prior to development of the zymogen granules (regulated secretion of enzymes) and the pancreatic duct.

The present experiments demonstrate the power of PCR in combination with classic embryological methods. The ability to detect low-level gene expression in small numbers of cells may allow us to resolve the long-standing issue in develop-

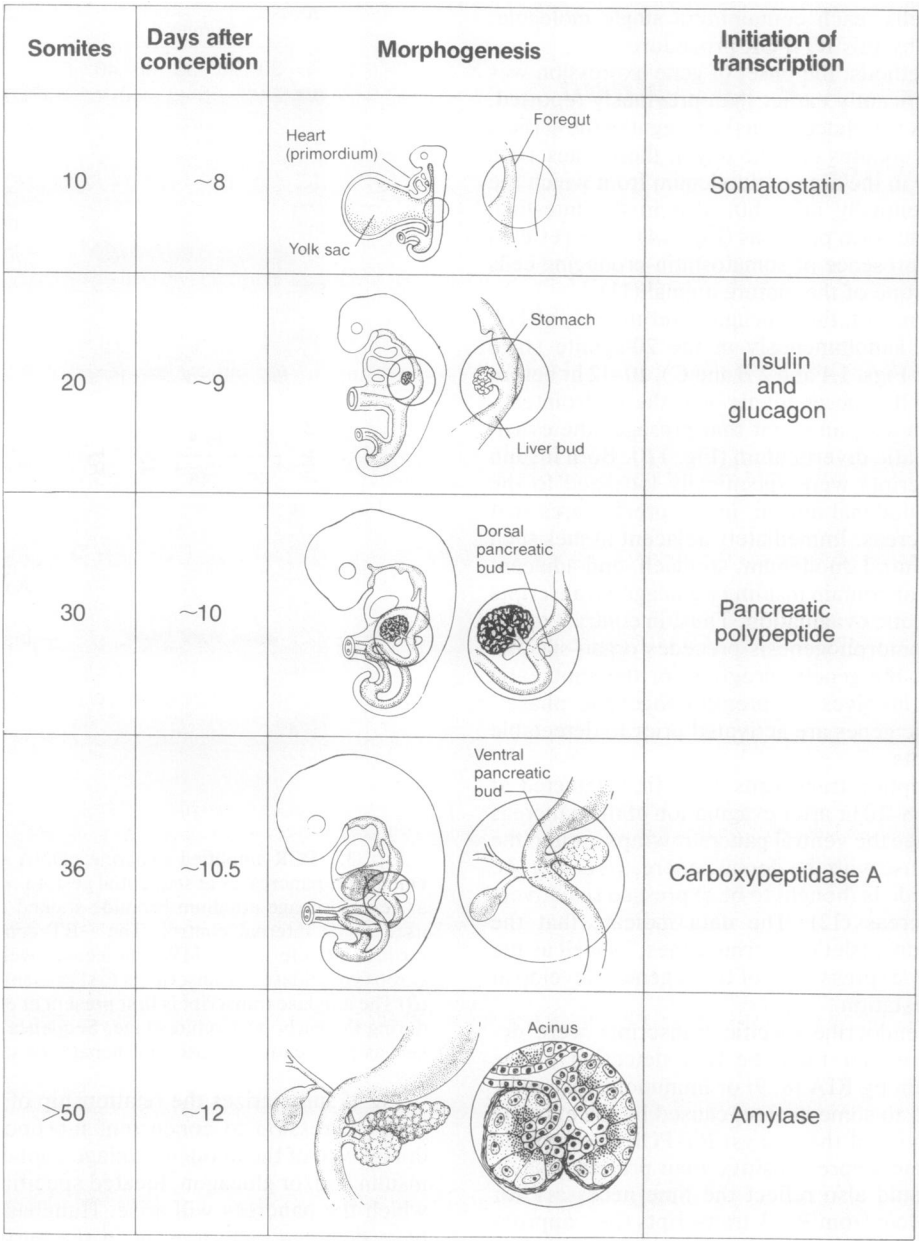

FIG. 4. Schematic diagram of the temporal relationship between the initiation of specific gene expression and morphogenic events in the developing mouse foregut and pancreas. The early somatostatin expression is not specific to the area of the foregut destined to form pancreas. Insulin and glucagon expression, however, in the 20-somite embryo, is specifically localized to the area of foregut destined to become pancreas. We now call this the premorphogenetic phase of pancreatic organogenesis. Pancreatic polypeptide transcription begins at 30 somites, about the same time that the ventral pancreas wraps around the intestine to approach the dorsal pancreas. Carboxypeptidase A and amylase are transcribed later, when acini are forming.

mental programming—i.e., whether differentiated cells derive from lineages or in colonies via inductive effects.

We thank L. DiLacio, R. Chadwick, and O. Venekei for technical assistance; D. Rappolee for advice on the RT-PCR technique; D. Standring for supplying the HBV plasmid; D. Hanahan for discussions and for advice on microscopy; J. Nielsen, M. Blanar, and T. Yoshida for important discussions; H. Debas for support; and M. German for continuous advice, insight, and criticisms.

1. Wessels, N. K. (1977) *Tissue Interaction and Development* (Benjamin, Menlo Park, CA).
2. Gilbert, S. (1989) *Developmental Biology* (Sinauer, Sunderland, MA), pp. 567–569.
3. Wessels, N. K. & Evans, J. (1968) *Dev. Biol.* **17**, 413–446.
4. Pictet, R. L., Clark, W. R., Williams, R. H. & Rutter, W. J. (1972) *Dev. Biol.* **29**, 436–467.
5. Han, J. H., Rall, L. R. & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 110–114.
6. Teitelman, G., Joh, T. H. & Reis, D. J. (1981) *Peptides* **2**, 157–168.
7. Sanders, T. G. & Rutter, W. J. (1974) *J. Biol. Chem.* **249**, 3500–3509.
8. Rall, L. B., Pictet, R. L., Williams, R. H. & Rutter, W. J. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 3478–3482.
9. Clark, W. R. & Rutter, W. J. (1972) *Dev. Biol.* **29**, 468–481.
10. Rappolee, D. A., Mark, D., Banda, M. J. & Werb, Z. (1988) *Science* **241**, 708–711.
11. Alumets, J., Sundler, F. & Hakanson, R. (1977) *Cell. Tissue Res.* **185**, 465–479.
12. Orci, L., Malaisse-Lagai, F., Baetens, D. & Perrelet, A. (1978) *Lancet* **ii**, 1200–1201.
13. DeGasparo, M., Pictet, R. L., Rall, L. B. & Rutter, W. J. (1975) *Dev. Biol.* **47**, 106–122.
14. Schibler, U., Pittet, A., Young, R., Hagenbuchle, O., Tosi, M., Gellman, S. & Wellaver, P. (1982) *J. Mol. Biol.* **155**, 247–266.
15. Alpert, S., Hanahan, D. & Teitelman, G. (1988) *Cell* **53**, 295–308.